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GRANT NUMBER DAMD17-98-1-8227

TITLE: Parity-Induced Protection Against Breast Cancer

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REPORT DATE: July 1999

TYPE OF REPORT: Annual

PREPARED FOR: Commanding General
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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DTIC QUALITY INSPECTED 4

REPORT DOCUMENTATION PAGE

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1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE July 1999		3. REPORT TYPE AND DATES COVERED Annual (1 Jul 98 - 30 Jun 99)	
4. TITLE AND SUBTITLE Parity-Induced Protection Against Breast Cancer				5. FUNDING NUMBERS DAMD17-98-1-8227	
6. AUTHOR(S) Celina M. D'Cruz					
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Pennsylvania Philadelphia, Pennsylvania 19104-3246				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES					
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited				12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 words) There is a strong correlation between a woman's reproductive history and her risk of developing breast cancer. In particular, epidemiological studies have shown that women who undergo an early first-full term pregnancy (FFTP) are at a much reduced lifetime risk of developing breast cancer as compared to women who never have children. This reduced susceptibility to breast cancer following an early FFTP has also been demonstrated in both rat and mouse models. Although this observation is well documented, the underlying molecular mechanism for parity-induced protection is not known. One model to explain this effect would be that the relative distribution of epithelial cell subtypes dramatically change as a result of pregnancy, lactation and regression, and give rise to a population of epithelial cells with a reduced tumor susceptibility. In a second model, it is hypothesized that systemic changes in hormone levels, and the indirect result of these changes on epithelial cell subtypes, may impact the susceptibility to tumorigenesis in the parous breast. To distinguish between these models, we have initiated a project to isolate molecular markers demonstrating differential expression between nulliparous and parous rat mammary glands. Preliminary results suggest that this is a valuable approach in which to characterize the cellular and molecular changes that occur as a result of parity.					
14. SUBJECT TERMS Breast Cancer Etiology, Endocrine, Carcinogenesis, Epidemiology				15. NUMBER OF PAGES 11	
				16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified 2	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited		

FOREWORD

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Celina M. D'Onofrio 8/2/99
PI - Signature Date

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Introduction:

The relationship between carcinogenesis and development in the breast is underscored by epidemiological studies in which breast cancer risk is associated with normal endocrine history. Reproductive events such as a late menarche or an early menopause are two events known to reduce a woman's exposure to ovarian hormones as well as her risk of mammary carcinogenesis[1-5]. In addition, an early first full-term pregnancy (or early FFTP) has been demonstrated to reduce a woman's lifetime risk of developing breast cancer as compared with women that never have a child[6-11]. To further support this observation, parity-induced protection has been observed in a Sprague-Dawley rat model as well[12,13]. Although parity-induced protection from breast cancer is well documented both in epidemiological and rodent studies, very little is known about the underlying molecular mechanism of this protective event. It has been hypothesized that the difference in tumor susceptibility between the nulliparous and parous breast may be due to an alteration in the distribution of epithelial cell subpopulations. Alternatively, parity-induced protection may result from systemic changes such as hormonal levels that indirectly effect the relative abundance of epithelial cell subpopulations in the breast. To address these two models, we are using PCR-based subtractive hybridization to isolate cDNA clones demonstrating differential expression between virgin and parous rat mammary glands. We have successfully generated non-overlapping, enriched populations of subtracted cDNA using both virgin and parous tissue as "tester" material. In addition, we have employed a secondary screening approach to improve the isolation of true positive clones. Based on the results of the secondary screening, we have sequenced for identification and confirmed expression by Northern hybridization of numerous clones. We have found that using this approach, we are able to isolate molecular markers that demonstrate differential expression and that of a subset tested, these markers also reflect changes in cell types within the breast by *in situ* hybridization.

SPECIFIC AIMS:

- 1). Identify molecular markers demonstrating parity-related changes in the rat breast.** We will identify genes that are differentially expressed between virgin and parous breast.
- 2). Characterize the temporal and spatial pattern of expression of molecular markers in the breast.** To better understand the regulation of the isolated cDNA markers within the breast, the expression of these genes will be analyzed at the relevant developmental time points by Northern and *in situ* hybridization.
- 3) Determine the expression of molecular markers with respect to parity-related reproductive histories.** We will use both Northern blot and *in situ* hybridization to analyze changes in the expression of molecular markers as a function of reproductive

BODY:

TASK 1. To identify molecular markers demonstrating parity-related changes in rat breast tissue: months 1-24

- **mate animals for virgin and parous breast tissue, harvest and purify total RNA: months 1-6**
- **generate cDNA libraries from virgin and parous rat breast tissue: months 6-12**
- **initiate differential screening of cDNA libraries: months 6-12**
- **initiate PCR-subtractive hybridization: months 6-12**
- **purify, sequence, and confirm expression of candidate markers: months 8-24**

Mate animals for virgin and parous breast tissue, harvest and purify total RNA: months 1-6

20 Sprague-Dawley female rats were received from Harlan Laboratories and housed 3 per cage with food and water ad libitum under viral and bacterial free conditions. 10 females were set-up at the age of 4-6 weeks with male rats. The males were removed prior to parturition and the females animals were allowed to lactate for 21 days. After this time, pups were weaned and the parous animals were allowed to regress for 28 days. At the designated time of sacrifice (approximately 15 wks), both parous animals and age-matched controls, that were housed in parallel, were euthanized by CO₂ inhalation and mammary glands 3-6 were harvested (excluding the lymph node in gland 4). Breast tissue was snap frozen and then homogenized in guanidine isothiocyanate and purified by ultracentrifugation through a CsCl gradient. Once total RNA was isolated and quantitated, purified poly(A) mRNA was isolated after 3 rounds of purification over an oligodT column. This additional purification of poly(A) was performed in addition to total mRNA purification as a recommendation for improving the outcome of the PCR-based subtractive hybridization technique.

Generate cDNA libraries from virgin and parous rat breast tissue: months 6-12
see below.

Initiate differential screening of cDNA libraries: months 6-12

This approach is valuable for identifying the differential expression of abundantly expressed cDNA. Results from our PCR-based subtractive hybridization suggest that this method identifies abundantly expressed messages as well as rare or low level messages. We are in the process of fully analyzing the subtractive hybridization data to determine whether screening of cDNA libraries would yield additional information or whether we should investigate microarray technologies.

Initiate PCR-Based Subtractive Hybridization

Using the poly(A) generated from a pool of 10 virgin and 10 parous rats, we made double-stranded cDNA, digested the cDNA with RSA 1, and ligated two sets of linkers as per Clontech's PCR-Select kit protocol. Following two rounds of subtractive hybridization with both virgin cDNA and parous cDNA as "tester" in the presence of

excess “driver”, differentially expressed messages were amplified by primer specific oligos to the linkers. In Figure 1. these pools of subtracted and amplified virgin or parous-specific cDNA are hybridized with parous markers to assay for enrichment. The results suggest that we have generated non-overlapping, enriched pools of cDNA from our parous “tester” sample. Unfortunately, we did not have a virgin-specific cDNA available to test for virgin enrichment. The Clontech kit was used in place of the Wang and Brown technique as previously proposed due to its relative success in other laboratories at the University of Pennsylvania School of Medicine.

Purify, Sequence, and Confirm Expression of Candidate Markers

The cDNA fragments were subcloned into pBluescript, transformed and plated to generate a collection of virgin and parous clones. 1000 virgin and 1000 parous clones were then gridded and duplicate colony lifts were prepared. Sets of filters were then hybridized with a virgin or parous cDNA probe. The duplicate filters were compared for differential hybridization. After this analysis, 100 clones were selected due to verified differential expression. Of these 100 clones, 97 were sequenced for identity. From the 97 sequenced cDNA, 91 matched to known sequences. Six sequences showed no significant homology to previously described sequences. Of the 97 genes identified, 50 were chosen as candidates for Northern hybridization. We were able to confirm 2 fold differences in 15 clones, 2 of which the identity is unknown. A panel of parous specific markers that have been confirmed by Northern hybridization are shown in Figure 2.

Task 2. To characterize the temporal and spatial expression of candidate molecular markers: months 12-36

- **generate parity-related developmental timepoints, prepare RNA and histological sections: months 12-18**
- **test temporal expression pattern of candidate markers: months 12-36**
- **perform *in situ* hybridization of interesting candidate markers: months 12-36**

Perform *in situ* hybridization of interesting candidate markers.

As a pilot test for large scale *in situ* hybridization of our candidate markers, we have tested 3 cDNA markers. In all cases, the molecular markers are identifying a cell population that is selectively expanded in the parous rat breast as compared to the virgin breast section.

KEY RESEARCH ACCOMPLISHMENTS:

- PCR-Select Hybridization enriched for differentially expressed messages.
- Several cDNA clones demonstrate differential expression between virgin and parous glands.
- *In situ* hybridization suggest that a subset of these markers tested verify an expansion of cell types in the parous breast as compared with the virgin breast.

REPORTABLE OUTCOMES:

L.A. Chodosh, C.M. D'Cruz, H.P. Gardner, S.I. Ha, S.T. Marquis, J.V. Rajan, D.B. Stairs, J.Y. Wang and M. Wang. *Cancer Research*(s)59,1756s-1772s,1999.

CONCLUSIONS:

In summary, we have isolated total RNA from the breasts of both virgin and parous rats. In addition we have purified poly(A) from this total RNA in order to improve the technical feasibility of PCR-Select. We have performed PCR-Based Subtractive Hybridization with both virgin and parous tester to identify tissue specific cDNA clones. From our pools of PCR-amplified products, we subcloned these fragments into pBluescript, sequenced for gene identity. We have been able to generate a panel of markers that demonstrate differential expression. In addition, several of these candidate molecules have been used as templates for *in situ* hybridization. From this analysis, we have found that this is a valuable approach to characterize the relative abundance of cell types in the breast. We will continue to further characterize the temporal and spatial pattern of the described markers, as well as correlate their expression with animals of a variety of reproductive backgrounds. In addition we are investigating newer technologies to identify differentially expressed cDNA markers, given that this approach appears to be a very fruitful means of understanding parity-induced protection.

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APPENDICES:

Figure 1

Figure 2

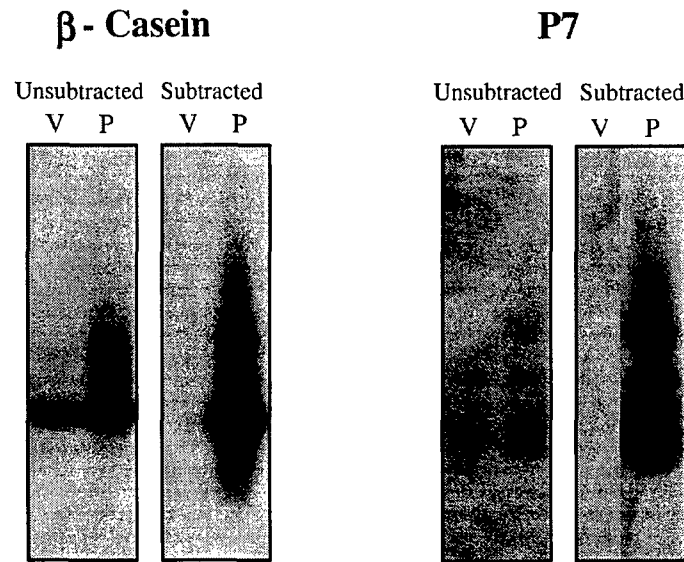


Figure 1. PCR-Select Enriches for Differentially Expressed Parous cDNA. Southern Blot hybridization of cDNA from unsubtracted or subtracted virgin and parous rat tissue hybridized with two previously identified parous cDNA markers. In the first panel, B-Casein, although present in unsubtracted virgin is subtracted out of the parous sample. In the second panel, clone P7 is detected at very low levels in the parous sample and is extensively enriched for in the subtracted and amplified parous cDNA pool.

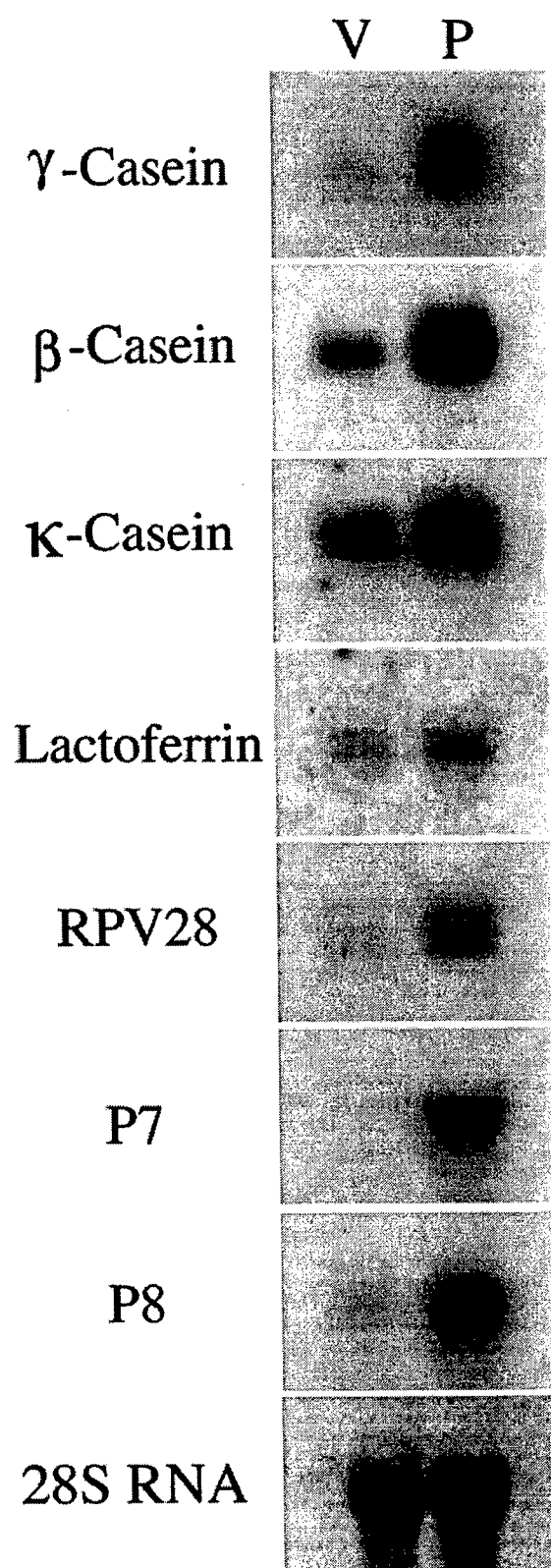


Figure 2. Northern blot hybridization of Parous Specific cDNA markers.